TOUCH-UP Gradient Amplification Method

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We report a unique amplification technique that works efficiently and specifically over a temperature range, rather than at one specific temperature, throughout the amplification process. As bisulfite-modified DNA is one of the difficult to amplify templates, we used this technique to amplify regions of promoter-associated CpG island for 11 genes using this template. This technique amplified specific products for every gene without requiring any optimization.

KEY WORDS: methylation specific PCR, DNA methylation analysis, COBRA, bisulphite modified DNA

INTRODUCTION

Bisulfite-modified DNA is the primary requirement for almost all DNA methylation analysis methods available today. Some of these techniques require amplification of the bisulfite-modified DNA to study methylation at specific loci within the genome. These include methylation-specific PCR,[1] combined bisulfite restriction analysis,[2] methylation-sensitive single nucleotide primer extension,[3] and bisulfite sequencing.[4] However, the successful amplification of bisulfite-modified DNA is often challenging. Conventional PCR is rarely used, and specialized modifications of PCR, such as touch-down or nested PCRs, are used frequently. Irrespective of the amplification method used, all require prior optimization of the PCR to determine suitable annealing temperatures. In addition, some methods require double amplification, using nested primers, which increases the chances of nonspecific product generation. Therefore, we report here a PCR amplification technique especially for bisulfite-modified DNA, which requires minimum optimization and produces specific products using a temperature range throughout the amplification process. This technique has been designated as Touch-Up (TU) gradient PCR.

To confirm the efficacy of this unique amplification technique promotor-associated CpG island of 11 genes—hexokinase-2 (*HK2*), metastasis suppressor-1 (*MTSS1*), supervillin (*SVIL*), tafazzin (*TAZ*), lim homeobox-9 (*LHX9*), basic helix-loop-helix family member e41 (*BHLHE41*), deiodinase, iodothyronine, type I (*DIO1*), PGD_2 synthase (*PTGDS*), DnaJ (Hsp40) homolog, subfamily C, member 15 (*MCJ*), epoxide hydrolase 1, microsomal (*EPHX1*), and NAD(P)H dehydrogenase, quinone 1 (*NQO1*)—they were amplified in IN699, a short-term cell culture derived from a pediatric glioblastoma multiform.

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MATERIALS AND METHODS

DNA Extraction and Bisulfite Modification

DNA was extracted from IN699 cells using a QIAamp DNA mini kit (Qiagen, West Sussex, UK), following the manufacturer's instructions. Cells were grown in Hams F10 nutrient mix (Invitrogen, Paisley, UK) with 10% FCS (Invitrogen) at 37°C in a non-CO_2 incubator before harvesting at passage 10. The quality and quantity of DNA were determined with a NanoDrop ND-2000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). Bisulfite treatment of DNA was performed using the EZ DNA Methylation-Gold kit, according to the manufacturer's instructions (Zymo Research, Cambridge, UK).

PCR Amplification

The primer sequences used in the current study were designed using MethPrimer and are as follows: *HK2* 5′-TTTTGGATTATTTGAGATTTTTGAGAT-3′ and 5′-ATTAGGGGATTGGTTTTTGGTT-3′ (292 bp); *MTSS1* 5′-GGGGTTTAAGGTATTGGTTGTAAGT-3′ and 5′-AAACTAACTTTCCCTCTCAATCTCC-3′ (208 bp); *SVIL* 5′-CGTTTGGTGGTTTAGTAGAGGGC-3′ and 5′-AAACTCGCGCGTCCCC-3′ (73 bp); *TAZ* 5′-GTTTATTTGGATTTTGGTTAGTAG-3′ and 5′-TACAACACCTCCCTATTATACAC-3′ (244 bp); *LHX9* 5′-AGGTTTTTTGTGTAGAGATGTGTT-3′ and 5′-AATACACCAAACTATCCTTCATACC-3′ (169

bp); *BHLHE41* 5′-GTTTGGAGTGAGAGTAAATTATTAG-3′ and 5′-TTAAAAAACCTTAAAAAAATCTATAC-3′ (180 bp); *DIO1* 5′-TGTATTAGTAGGTAAAGAAAAGAGTGT-3′ and 5′-CTCCCAAATAACTAAAATTACAAAC-3′ (206 bp); *PTGDS* 5′-TTTGTTAGGGGAAGGGTATTTTT-3′ and 5′-TATCCTAACACCCAAATATAAAAC-3′ (300 bp); *MCJ* 5′-AAGTATATAAAGTTTTTTGAGGGTT-3′ and 5′-CAAAACTCACCAATCTCTACTAATC-3′ (401 bp); *EPHX1* 5′-TATTGGGGGAAGGAGTTTGTAG-3′ and 5′-AAACAACCATATTACTCACA3CAAAAC-3′ (265 bp); *NQO1* 5′-GTTTTTGTAGGTTGTTTATTTTAAA-3′ and 5′-CCTCCACAAACACCAATACTC-3′ (258 bp).

Bisulfite-modified DNA (2 μl) was amplified using gene-specific primers and 1 U HotStarTaq DNA polymerase (Qiagen) in a 20-μL reaction volume, according to the manufacturer's instructions. HotStarTaq DNA polymerase required an initial activation step of 95°C for 15 min before the start of the cycling. The cycling conditions used for each of the protocols followed are: conventional PCR (40 cycles; 95°C for 30 s, 48°C for 30 s, and 72°C for 1 min) and touch-down PCR [initial 10 cycles—95°C for 30 s, 58°C for 30 s (−1°C every cycle), 72°C for 1 min], followed by 40 cycles of 95°C for 30 s, 48°C for 30 s, and 72°C for 1 min. However, in TU gradient PCR, a 10-cycle loop, consisting of 95°C for 30 s, 48°C for 30 s (+0.5°C every cycle), and 72°C for 1 min, was carried out. The 10-cycle loop was repeated five times (50 cycles). The amplification products were separated on 2.5% agarose gels and compared with O'GeneRuler 100 bp DNA Ladder (Fermentas, York, UK).

RESULTS AND DISCUSSION

The TU gradient amplification protocol (Fig. 1A) was compared with conventional and touch-down PCRs (Fig. 1B and C, respectively). In this comparative analysis, con-

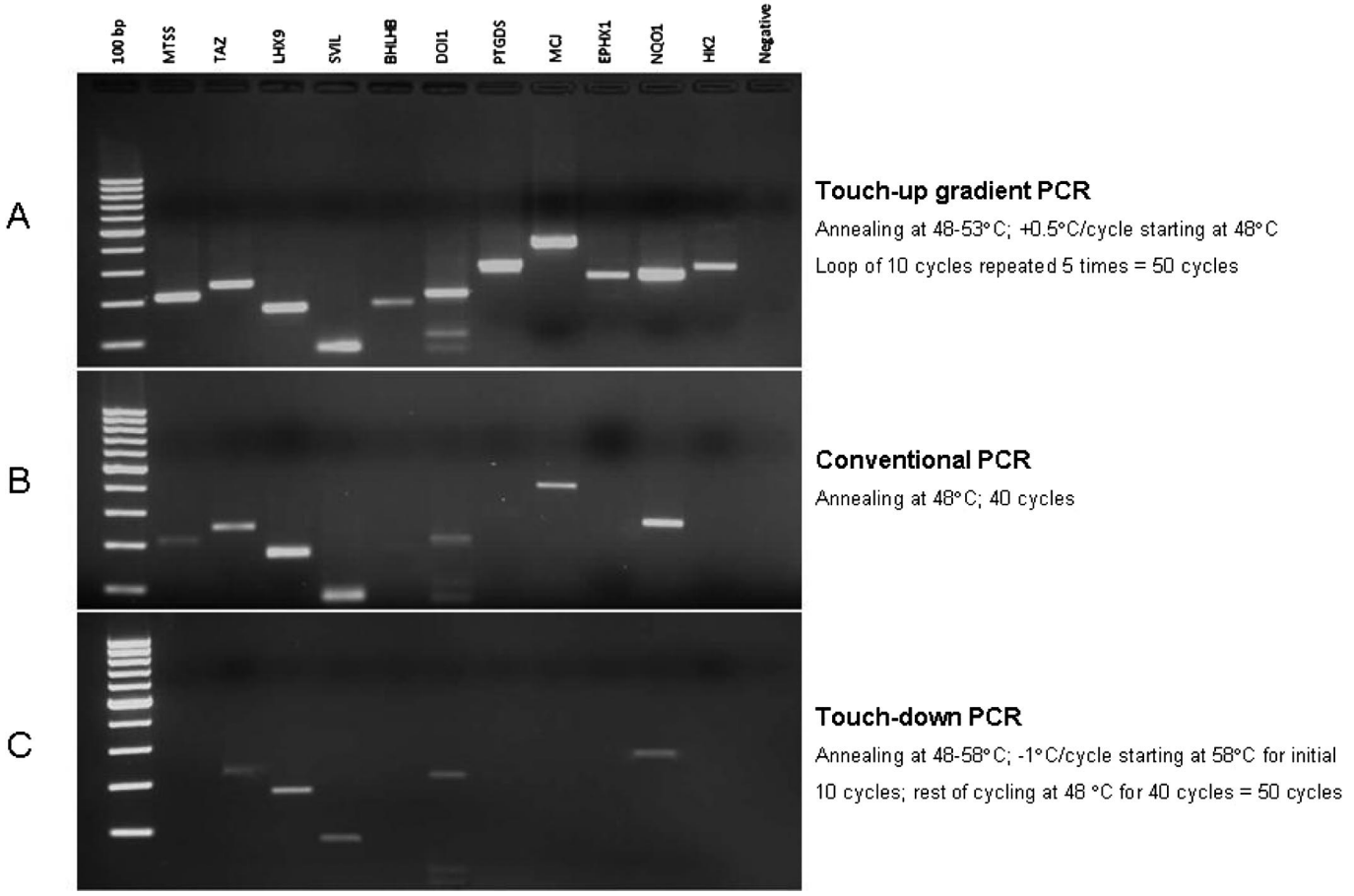

FIGURE 1

Comparison of touch-up gradient, conventional, and touch-down PCRs to amplify promoter associated CGO of genes *MTSS1, TAZ, LHX9, SVIL, BHLHE41 (BHLHB), DIO1, PTGDS, MCJ, EPHX1, NQO1,* and *HK2*. A: touch-up gradient protocol employing 48–53°C temperature range. Briefly, the 10 cycle amplification loop starting at 48°C and increasing by 0.5°C every cycle was employed and this loop was repeated five times. B: conventional amplification using an annealing temperature of 48°C for 40 cycles. C: touchdown protocol employing 48–58°C temperature range followed by 40 cycles at 48°C.

ventional and touch-down PCRs produced specific products for seven and five out of 11 genes, respectively, while the touch-down gradient PCR produced specific amplification products for the 11 genes analyzed. For *DOI1*, all of the three amplification protocols produced more than one product and therefore, could be accounted for by the primer sequence. In spite of using the same quantity of template DNA for all of the amplification reactions, the products generated by the touch-down gradient PCR were more intensely visualized by ethidium bromide staining compared with other protocols. Unlike the conventional PCR (40 cycles), touch-down and TU gradient PCRs have similar numbers of cycles, i.e., 50. By using the same sets of primers for all three amplification methods for the 11 genes analyzed, we have demonstrated that successful amplification was a result of the type of cycling used rather than the quality of the primers or number of cycles performed.

Touch-down PCR uses the principle that performing early PCR cycles at an annealing temperature, which is higher than the target optimum, and gradually reducing it to target optimum result in the reduction of nonspecific amplification.[5] The remainder of the cycling is performed later at the optimum annealing temperature. In this paper, we report an alternative and exact-opposite cycling mechanism, which we designate as TU gradient PCR. In this cycling protocol, we start the initial cycling a few degrees below the target annealing temperature (e.g., 48°C) and increase it gradually, by 0.5°C/cycle for the first 10 cycles. At the end of this initial cycling, the loop of 10 cycles is repeated four more times. The initial cycles of the loop provide lenient conditions for the amplification to occur, whereas the later cycles enable more specific amplification. The leniency and stringency at different parts of the gradient loop are repeated without compromising the specificity of the reaction. This type of cycling has produced specific products the first time for all of the genes assessed. We also assessed the specificity of this protocol by repeating the 10-cycle loop for nine times (90 cycles), and the amplified products still remained specific (data not shown). Therefore, we propose that the TU gradient PCR will be useful for templates that are difficult to amplify and require extensive optimization, e.g., bisulfite-modified DNA. Future work on TU gradient PCR would be to ascertain its role in amplifying other difficult templates, such as GC-rich regions of genomic DNA and whole genome amplifications.

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